An Improved Quantitative Method for Volatile Phenols

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A method has been developed for the quantitative analysis of volatile phenols in crude organic mixtures. The analysis involves a one-step fractionation by thin-layer chromatography and determination by gas–liquid chromatography. The procedure is reproducible and has been applied to the determination of phenols in tobacco smoke condensate.

THE PHENOLS OF TOBACCO SMOKE condensate have been implicated as potent promoters in mouse-skin carcinogenesis (*1*) and phenols in general contribute to the toxicity and pollution in industrial environments. A rapid, but still accurate method for the determination of phenols would find wide application not only in the analysis of crude industrial mixtures, but also in complex plant extracts.

After extensive reviews were made of the literature on quantitative methods for phenols, several points have become apparent. Initially, colorimetric procedures were employed, whereas lately, gas–liquid chromatography (GLC) has become the final tool. Phenolics have been qualitatively or quantitatively determined as colored derivatives on chromatographic media or in solution by such reagents as: diazotized sulfanilic acid (*2*), diazotized *p*-nitro aniline (*3*), *p*-dimethylaminobenzaldehyde (*4*), and 4-aminoantipyrene (AAP) (*5*). However, most of these chromogenic analyses suffer from a serious deficiency: para-substituted phenols do not react, or only partially, and thus cannot be determined in this manner. Many papers have appeared on the application of GLC to the resolution and quantitative analysis of mixtures of volatile phenols. GLC offers the advantages of sensitivity and convenience. However, GLC requires a relatively "clean" mixture for analysis. This in turn requires a preliminary purification by other chromatographic or extraction methods. The conventional isolation procedure usually involves aqueous alkaline extraction of the crude organic mixture, solvent partitions, and steam distillation. Such extensive techniques usually yield quite low and inaccurate results because of losses due to volatility or due to differences in distribution coefficients between extracting solvents. Attempts to correct this deficiency through the use of an internal standard have not succeeded. Thus, the addition of a trimethyl phenol to correct for losses of a xylenol is not appropriate, because of difference in behavior of one substituted phenol as compared to another. The ideal solution would be to use a labeled compound as an internal standard to correct for losses of the identical unlabeled compound. Since such a procedure is not always practical, another approach to the problem of phenol determination seemed desirable. We have taken a step in this direction by developing a quantitative, two-step method for volatile phenols, involving thin-layer chromatography (TLC) and GLC.

EXPERIMENTAL

Thin-Layer Chromatography. TLC was carried out on 20 × 20 cm plates, coated to a thickness of 500 μ, using a Desaga (Mention of an item does not imply its endorsement by the Department over similar products not mentioned.) applicator, with a mixture (1:1) of Brinkmann Silica Gel G and Brinkmann Cellulose MN powder, mixed in an electric blender (*6*). Plates were air-dried at room temperature for at least 6 hours and developed with cyclohexane (spectro grade)–ethyl acetate (redistilled) (5:1, v/v). Phenol-containing mixtures were applied as solutions in acetone, ethyl ether, cyclohexane, or ethyl acetate. Solvents such as ethyl and methyl alcohol affected the quantitative recovery, retaining part of the phenols at the origin. A solution of smoke condensate was prepared in the following manner. A condensate sample of about 0.4 gram was removed from a 1-kg batch of crude smoke condensate. The sample was dissolved in acetone and centrifuged. The small amount of precipitate was again washed thoroughly with acetone and centrifuged. The solutions were combined and adjusted in volume such that the final concentration of condensate was about 100 mg/ml. Usually 250 μl, an amount equivalent to 1.2–1.5 cigarettes, was then applied to the TLC plate. (The condensate was obtained from cigarettes smoked under established parameters: a puff volume of 35 ml, duration of 2 seconds, and butt length of 28 mm. The smoke was condensed in dry-ice traps and washed out with acetone.) A Radin-Pelick TLC sample streaker was used to apply a uniform band of standards or known solutions across 14 cm of the plate. After an appropriate space, another 2 cm of the unknown solution (or standards) of comparable concentration was applied as reference. After development, the main portion of the plate was covered with another glass plate and the reference phenols were visualized by spraying with diazotized *p*-nitroaniline, followed by 10% aqueous sodium hydroxide. The corresponding zone of unknown phenols (at R_f of about 0.7) was scraped from the TLC plate with a razor blade and transferred to a microfilter funnel with a sintered glass disk (porosity 0.06–0.08 μ). The adsorbent was washed consecutively with 4 ml and 14 ml of a mixture of ethyl acetate (EA)–cyclohexane (CH) (1:1, v/v). The first 4 ml of wash solution, which contains about 70% of the phenols, was set aside. The second extracting solution was reduced by a stream of nitrogen gas to about 1 ml. Both extracts were combined and further reduced to a final volume of 2 ml.

Gas–Liquid Chromatography. GLC was conducted on 6-foot, $^1/_8$-inch stainless steel columns packed with 10% UC-W98 silicone on 80/100 mesh chromosorb W. An F & M 5750 gas chromatograph, equipped with flame ionization detectors, was employed. The oven was programmed at 1°/min. The detector and injection port were kept at 195 and 250 °C, respectively. The GLC procedure was calibrated by analysis of a mixture of the following pure standards in CH: phenol, *o*-cresol, *m*-cresol, *p*-cresol, 2,4-xylenol- and *p*-ethyl phenol (in a weight ratio of 5:1:1:1:1:1). Calibra-

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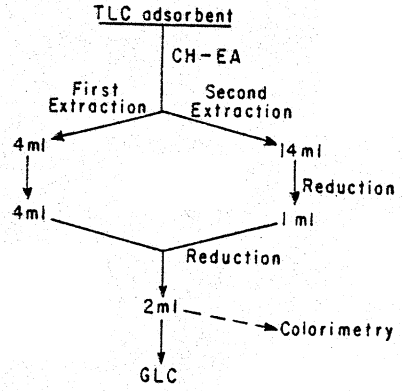

Figure 1. Solvent extraction procedure for phenols from TLC adsorbent

Table I. Recovery of Standard Volatile Phenols

Compound	Recovery,[a] %	Std dev, %
Phenol	94.4	3.4
o-Cresol	93.0	0.3
m-Cresol	95.1	2.0
2,6-Xylenol	89.3	1.4
2,5-Xylenol	88.4	2.5
3,4-Xylenol	98.1	0.5
o-Ethyl phenol	95.4	0.8
p-Ethyl phenol	94.8	0.7

[a] An arithmetic mean value for eight determinations.

tion curves for each component were plotted, with concentration *vs.* peak area, calculated by the triangulation method. Detector response was linear with concentration, but varied slightly from day to day.

Colorimetry. The colorimetric AAP method for total volatile phenols of tobacco smoke, as presented by G. Lorentzen and G. Neurath (*5*) and confirmed by others (*7*), has been adopted without change in this study. Samples for colorimetric determination were steam distilled in a single-unit, micro Kjeldahl apparatus (*8*). Calibration curves were prepared for individual phenols as well as for a mixture simulating the phenolic distribution in tobacco smoke condensate.

RESULTS AND DISCUSSION

Our conjunctive interests in TLC and the phenolic constituents of tobacco leaf have led to development of a method for volatile phenols in crude mixtures. Such mixtures usually contain various types of acidic or phenolic constituents. Our objective in TLC was to separate the volatile, monohydroxy phenols in a single band away from other phenolic and polar material. After numerous experiments with various adsorbents, combinations of adsorbent and different solvent systems, we adopted a 1 to 1 mixture of silica gel and cellulose with CH–EA (5:1, v/v) as the solvent system. After separation of the volatile phenols from the other materials, they were extracted from the TLC adsorbent and subjected to further separation and quantitative analysis by GLC.

The method was first examined for percentage recovery of standard compounds. Thus, a mixture of known, volatile phenols was subjected to the above procedure and the analytical results were compared to starting measured amounts. Briefly, the analysis was conducted in the following manner. The measured mixture of knowns is applied to the TLC plate with a small amount of the same phenols applied separately at the edge. After chromatography, the position of the reference compounds on the edge was visualized with diazotized *p*-nitroaniline spray reagent. The corresponding zone of standard compounds was scraped from the plate and the phenols were eluted from the adsorbent with small volumes of a mixture of CH–EA (1:1, v/v). The resulting solution can be reduced in volume under nitrogen or used directly for GLC, depending upon the sensitivity of the detector. The GLC separation was carried out on columns of UC-W98, using a flame ionization detector. Quantitative calculations for each phenol were made from peak areas based on detector response for each compound. The separation of *m*- and *p*-cresol could not be accomplished by this column and these compounds were determined jointly. Table I lists the arithmetic mean for the per cent of recovered compound, together with the standard deviation from the mean for eight common, volatile phenols. It should be noted that 88–98% of the phenols are recovered, but specifically, that each phenol was lost to a different extent, which is characteristic of that compound. The greater losses of 2,6- and 2,5-xylenol may be due to decreased hydrogen bonding as a result of steric hindrance by adjacent methyl groups, leading to greater loss during TLC.

The next important parameter that was examined was the choice of TLC extracting solvent and the final volumes to which it could be reduced. The reduction in volume is one step where losses due to volatility are quite large but still can be restricted. Solvent removal can be accomplished by several methods, including reduction on a rotary evaporator under vacuum, by a stream of nitrogen at room temperature, or by distillation in a spinning band column. It was desirable to ascertain which procedure gave the lowest losses. Phenol was chosen for this study and the percentage of recovery was checked by both GLC and colorimetry (AAP). The results from both determinations were in close agreement. Relatively concentrated solutions of phenol (1 mg/ml) were prepared in ethyl ether or cyclohexane, solvents that have been employed in other phenol determinations, and 40 ml of solution were reduced to 5 ml by each of the three methods. Recoveries ranged from 40% for cyclohexane solutions on a rotary evaporator to 85% for ether solutions reduced by nitrogen. Reduction by spinning band column gave 70% and 80% recovery for cyclohexane and ether, respectively. It was concluded that nitrogen removed solvent vapors as well as any of the other methods, and this procedure was later used for all determinations. It should be noted, however, that the concentrations of our extracting solutions were much smaller (about 15 μg/ml) and it was shown that the reduction was not accompanied by any measurable losses. The extracting solvent that was finally chosen was a mixture of CH–EA (1:1, v/v), mainly because it quantitatively removed the phenols from the TLC adsorbent. The final procedure that was adopted for extracting the phenols from the TLC band is that shown in Figure 1.

Because of our research interests, we have applied this method to the analysis of complex tobacco extracts and tobacco smoke condensates. We will present our more significant results for condensate as proof of the reproduc-

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ibility of the method. Although numerous methods (*5, 9–14*) have been proposed for the analysis of volatile phenols of smoke condensate, the lengthy isolation procedures introduce losses that have been difficult to correct. In applying our method to smoke condensate, we wished to separate only the steam-volatile, monohydroxy phenols from the remainder of the crude, highly complex mixture of organic compounds. [More than 1000 constituents have been identified (*15*).] Thus a measured amount of condensate, about 30 mg, equivalent to about 1.5 cigarettes, was applied to a TLC plate and a mixture of standard volatile phenols was separately added on the edge as reference. Preferably, condensate solution of comparable concentration can be used as reference, as this will indicate the width of the phenolic band that is to be removed. After development, the phenol-containing zone of condensate was scraped from the plate, the compounds were eluted, and were analyzed by GLC. Results are presented in Table II in terms of micrograms of phenols per milligrams of condensate and per cigarette. Since random samples were repeatedly withdrawn from the same batch of condensate, the resulting values should be quite representative. These results are on condensate that had been stored in the refrigerator for several months and are presented only to illustrate the precision of the method, as reflected by the low values for the average deviation.

In order to determine whether or not the total calculated value (151.9 μg/cig) represents most of the volatile phenols in this condensate, we have steam distilled an aliquot of it. The distillate was used directly, without extraction, to determine the total amount of steam volatile, non-para-substituted phenols by the AAP method. A value of 126.5 μg/cig was obtained. This represents 83% of the total GLC-determined phenols, as the AAP method does not determine para-substituted phenols. Subsequently, the monophenolic band from TLC of an identical aliquot was extracted and the phenols again determined colorimetrically, giving a value of 129 μg/cig, in fine agreement with the above result. It was therefore concluded that this method yields precise results for the volatile phenols of tobacco smoke condensate.

A final point that had to be examined was whether or not the condensate phenols behaved in the analysis in a manner similar to standards. This was accomplished by adding a known amount of standard phenols to a given weight of condensate in solution. The mixture was carried through the TLC–GLC procedure and the results, corrected for the added standard, were compared with the previously established condensate values (Table II). Numerous such determinations were carried out and the results were similar, even when standards were applied on top of condensate on a TLC plate. The analytical data for one such determination is presented in Table III. Comparison of columns A and E shows good agreement between the values and leads to the conclusion that there is no drastic difference in the behavior of condensate and standard phenols.

This method has also been applied to analysis of phenols and phenolic acids of tobacco leaf extracts. Results of this work will be presented in a subsequent report.

Table II. Phenols of Smoke Condensate

	Content, μg/mg	Content,[a] μg/cig	Av dev, μg/cig
Phenol	3.19	63.7	0.6
o-Cresol	0.89	17.8	0.4
m + *p*-Cresol	2.22	44.4	2.2
2,4-Xylenol	0.46	9.2	0.5
p-Ethyl phenol	0.83	16.7	0.83
Total		151.9[b]	

[a] Average of five determinations, using the empirical relationship 20 mg of condensate = one cigarette.

[b] Compared to 126.5 μg/cig by colorimetry on steam distillate of condensate; para-substituted phenols not measured by AAP method.

Table III. Condensate with Added Standard Phenols

	A, μg/mg	B, μg	C, μg	D, μg	E, μg/mg
Phenol	3.19	120.0	49.0	71.0	2.89
o-Cresol	0.89	32.4	10.9	21.5	0.88
m + *p*-Cresol	2.22	79.3	23.2	56.1	2.28
2,4-Xylenol	0.46	22.3	11.4	10.9	0.45
p-Ethyl phenol	0.83	29.5	9.3	20.2	0.82

A. Established values for this condensate (Table II).
B. Total for added standards and condensate. (24.6 mg condensate).
C. Added standards corrected for losses (Table I).
D. Due to 24.6 mg of condensate.
E. Net values for phenols in condensate.

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